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MECHANISMS OF RESISTANCE

Zinc Acetate Potentiates the Action of Tosufloxacin against *Escherichia coli* Biofilm Persisters

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ABSTRACT Formation of bacterial biofilms is a major health threat due to their high levels of tolerance to multiple antibiotics and the presence of persisters responsible for infection relapses. We previously showed that a combination of starvation and induction of SOS response in biofilm led to increased levels of persisters and biofilm tolerance to fluoroquinolones. In this study, we hypothesized that inhibition of the SOS response may be an effective strategy to target biofilms and fluoroquinolone persister cells. We tested the survival of *Escherichia coli* biofilms to different classes of antibiotics in starved and nonstarved conditions and in the presence of zinc acetate, a SOS response inhibitor. We showed that zinc acetate potentiates, albeit moderately, the activity of fluoroquinolones against *E. coli* persisters in starved biofilms. The efficacy of zinc acetate to increase fluoroquinolone activity, particularly that of tosufloxacin, suggests that such a combination may be a potential strategy for treating biofilm-related bacterial infections.

KEYWORDS SOS system, biofilms, persisters, tosufloxacin, zinc acetate

Bacterial chronic infections are a serious health care problem that is often associated with the formation of highly antibiotic-tolerant biofilms (1). Although multifactorial, this high level of tolerance is at the origin of most of the treatment failure of biofilm-associated infections and is mainly due to the high frequency of persister bacteria (persisters) in biofilms compared to that of planktonic bacteria (2). Persister cells are phenotypic variants that can revert to wild-type (wt) antibiotic susceptibility and are considered to be the source of relapse of biofilm-related infections and reinfection (3). Therefore, there is a strong need to develop effective treatment strategies against biofilm persisters.

Escherichia coli is a versatile species that comprises commensal strains but also pathogenic strains frequently involved in a broad spectrum of intra- and extraintestinal diseases (4). Uropathogenic *E. coli* bacteria are the most prominent causative agents of urinary tract infections (UTIs) (5). In the case of UTIs, most relapse events are due to regrowth of *E. coli* persisters after treatment (6). Recently, the fluoroquinolone antibiotic tosufloxacin and the antimicrobial peptide colistin were reported as effective against uropathogenic *E. coli* persisters obtained from planktonic stationary-phase populations (6). In addition, tosufloxacin has also been reported to be highly active against planktonic *Staphylococcus aureus* persisters (7). However, since the levels and nature of persisters may differ greatly between planktonic and biofilm cells, the activity of antibiotics against persisters should also be evaluated under biofilm conditions.

We previously showed that starvation stress experienced by biofilm bacteria increases the level of tolerance to fluoroquinolone ofloxacin in *E. coli* biofilms (8). This increased tolerance of biofilms to ofloxacin upon starvation is dependent on the

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presence of a functional bacterial SOS response (8). Tolerance to ofloxacin treatment in the stationary phase was also shown to require activation of the SOS response during the posttreatment recovery phase (9). In addition to tolerance, the SOS response has been shown to be involved in horizontal gene transfer, emergence of antibiotic resistance, and toxin production (10). Several candidate inhibitors of the SOS response have been reported, including zinc, for which the molecular details of activity have been well described (11–15). Zinc inhibits the SOS response by interfering with the RecA ATP-binding site, which is essential for RecA activation (14). The role of SOS response in persister generation therefore raises the possibility of using the SOS response as a target to reduce fluoroquinolone persisters and the emergence of biofilm tolerance to antibiotics in clinical settings (11, 16).

In the current study, we hypothesized that inhibition of the SOS response may be an effective strategy to eliminate planktonic and biofilm fluoroquinolone persisters. After evaluating the effectiveness of various antibiotics against *E. coli* persisters in starved and nonstarved biofilms, we used zinc acetate as an SOS response inhibitor and showed that zinc acetate can be used as an adjuvant to increase fluoroquinolone activities, including that of tosufloxacin, against persisters. This study supports the possibility to use SOS response inhibitors to improve antibiotic activity against biofilm-related infections and persisters.

RESULTS

Starvation enhanced *E. coli* biofilm antibiotic tolerance in both SOS-dependent and SOS-independent manners. We previously demonstrated that starved *E. coli* biofilms display increased tolerance to ofloxacin (8). We wondered whether starvation also promoted increased tolerance when biofilms were treated with other fluoroquinolones and other classes of antibiotics. We assessed the efficacy of various quinolones/fluoroquinolones (nalidixic acid, ofloxacin, norfloxacin, ciprofloxacin, and tosufloxacin), penicillin (ticarcillin), aminoglycosides (amikacin and gentamicin), chloramphenicol, tetracycline, rifampin, and a polypeptide (polymyxin B). Among the different antibiotics tested, polymyxin B was the most effective antibiotic against *E. coli* TG1 biofilms (whether starved or not), with an observed 6-log reduction in survival (Fig. 1).

The fluoroquinolones ofloxacin, norfloxacin, ciprofloxacin, and tosufloxacin were also relatively efficient at reducing *E. coli* TG1 survival in nonstarved biofilms with tosufloxacin as efficient as ciprofloxacin and norfloxacin. Starvation led to significantly increased tolerance of *E. coli* TG1 biofilms to some antibiotics, including ofloxacin, ticarcillin, ciprofloxacin, norfloxacin, tosufloxacin, nalidixic acid, amikacin, and gentamicin (Fig. 1). In starved biofilms, tosufloxacin was the most efficient fluoroquinolone at killing *E. coli* TG1 biofilms. The increased survival of *E. coli* TG1 in starved biofilms treated with fluoroquinolones was abolished in the SOS response mutant (*lexAind3* mutant). In contrast, the SOS response did not appear to be involved in the enhanced tolerance of the starved biofilms treated with the other antibiotics, ticarcillin, nalidixic acid, amikacin, and gentamicin. Time-kill curves of 24-h *E. coli* TG1 biofilms treated by ofloxacin or tosufloxacin showed a typical persistence biphasic shape with a persister plateau reached after 20 h of treatment and an increased SOS-dependent tolerance in starved biofilms (Fig. S1).

The SOS response is required for increased ofloxacin tolerance of starved biofilms during the posttreatment recovery phase. To firmly correlate the level of tolerance of the starved biofilms treated with fluoroquinolones with the level of induction of the SOS response, we used *lexA* variant strains that display different abilities to induce the SOS response (17). Inactivation of the self-cleavage of LexA (S119A) totally inhibits the SOS response. Mutations in LexA can either decrease (G80A) or increase (E86P) the rate of self-cleavage relative to that of the wild-type strain (MMR102_F′tet) and thus affect the level of SOS induction. S119A, G80A, wild-type, and E86P strains all display successively increased efficacy in SOS response (17). Tolerance of the stationary phase of liquid culture (Fig. S2) and starved biofilms (Fig. 2) of the

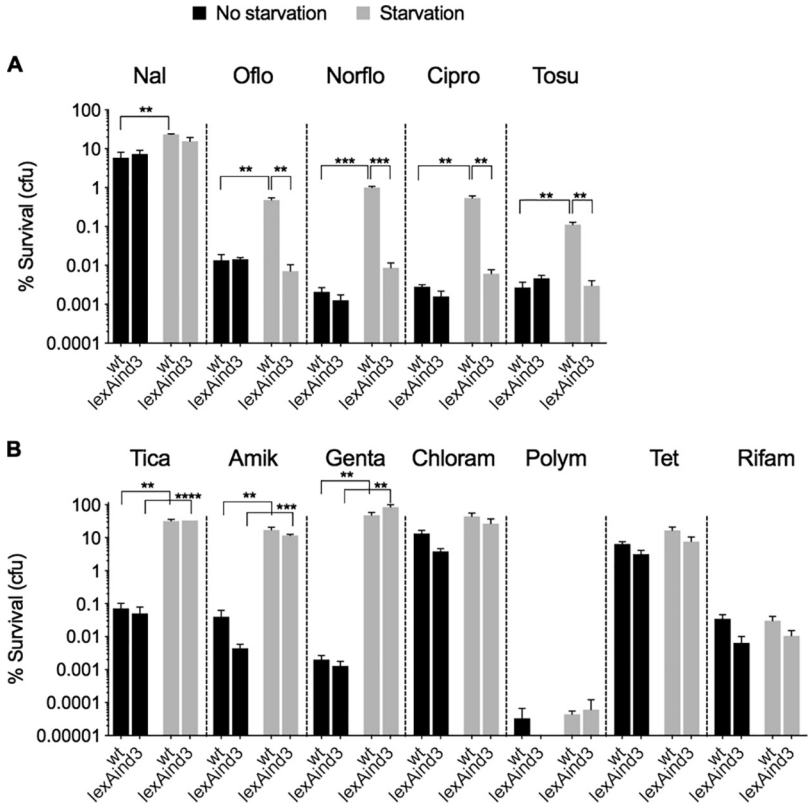

FIG 1 Survival rates of starved and nonstarved biofilms of *E. coli* K-12 TG1 and TG1 *lexAind3* (SOS response-defective mutant) strains when treated with antibiotics. Biofilms of *E. coli* TG1 and TG1 *lexAind3* (TG1 *lexAind3* Tet^r for all antibiotics with the exception of tetracycline and TG1 *lexAind3* Cm^r for tetracycline) were grown for 24 h in M63B1Gluc and treated for a period of 24 h with one of several antibiotics in M63B1 Gluc medium (black bars; nonstarved) or M63B1 medium without glucose (gray bars; starved). (A) Nalidixic acid (Nal; 640 mg/liter, 80× MIC), ofloxacin (Oflo; 5 mg/liter, 80× MIC), norfloxacin (Norflo; 5 mg/liter, 80× MIC), ciprofloxacin (Cipro; 2.5 mg/liter, 80× MIC), and tosufloxacin (Tosu; 5 mg/liter, 80× MIC). (B) TicarcilIn (Tica; 100 mg/liter, 100× MIC), amikacin (Amik; 320 mg/liter, 80× MIC), gentamicin (Genta; 40 mg/liter, 80× MIC), chloramphenicol (Chloram; 160 mg/liter, 80× MIC), polymyxin B (Polym; 80 mg/liter, 80× MIC), tetracycline (Tet; 40 mg/liter, 80× MIC), and rifampin (Rifam; 1280 mg/liter, 80× MIC). Surviving cells were quantified by viable cell counts. Percentage (%) of survival represents CFU count of viable cells after 24 h of treatment compared to that of untreated biofilms prior to the addition of the antibiotics. Each percentage (%) value is the mean plus or minus standard error of the mean (SEM) from at least three replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

different strains to ofloxacin directly correlated to the level of functionality of the SOS response. However, the survival rates of the *lexA* variants in the stationary phase of liquid culture (Fig. S2) and starved biofilms (Fig. 2) treated by ticarcillin were completely independent of the SOS response.

It was previously shown that upon fluoroquinolone treatment, the survival of planktonic stationary-phase persisters requires SOS-induced DNA repair during the posttreatment recovery period (9). To further clarify SOS response requirements for survival of persisters in starved biofilms upon fluoroquinolone treatment, we used plasmids expressing *recA*, the main SOS response activator gene, or *lexAind3* (or *lexA3*), a dominant negative *lexA* allele, to activate or block the SOS response in a timely manner, respectively (9). Ofloxacin persisters were less abundant in the *recA*-deficient strain compared to those in the wild-type strain (Fig. 3). In this *recA*-deficient mutant, induction of RecA in *trans* during the biofilm formation, ofloxacin treatment, and recovery phases was sufficient to restore levels of persisters comparable to those of wt bacteria and *recA*-deficient mutant bacteria with *recA* expressed in *trans* during all phases in starved biofilms (Fig. 3). With impaired SOS induction, which was achieved with *lexA3* expression, we observed a decreased survival compared to that in unin-

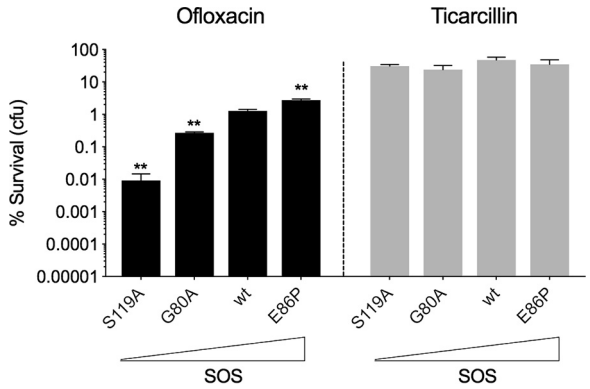

FIG 2 Survival rate of *E. coli lexA* variants treated by ofloxacin or ticarcillin in starved biofilms. Biofilms of *E. coli* were grown 24 h in M63B1Gluc medium and treated for a period of 24 h in M63B1 with ofloxacin (5 mg/liter, 80× MIC; black histograms) or ticarcillin (100 mg/liter, 100× MIC; gray histograms). Surviving cells were quantified by viable cell counts. All strains carried the F′tet conjugative plasmid. *lexA* variants S119A and G80A, wt MMR102, and *lexA* variant E86P strains have gradually increased capacity to induce the SOS response. Percentage (%) of survival represents the CFU count of viable cells after 24 h of treatment compared to that of untreated biofilms prior to addition of antibiotics. Each percentage (%) value is the mean plus or minus standard error of the mean (SEM) from at least three replicates. Asterisks indicate values significantly different from that obtained from the wild-type cells (MMR102_F′tet). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

duced controls (Fig. 3). This held true with *lexA3* induction during biofilm formation only, ofloxacin treatment only, recovery only, and any combination of the three periods of treatment (Fig. 3). Overall, the results indicated that induction of the SOS response before, during, or after fluoroquinolone treatment during recovery was sufficient to increase persister survival.

Zinc acetate potentiates the activity of fluoroquinolones against persisters in starved *E. coli* biofilms. To validate the activity of zinc acetate as an SOS inhibitor in our current study settings, we monitored SOS induction in planktonic bacteria upon ofloxacin treatment through the expression of *sulA* in the presence and absence of zinc acetate (Fig. S3). Increasing concentrations of zinc acetate were used, ranging from 0 to 1 mM, which was below the 1.5 mM MIC of zinc acetate in *E. coli* (Fig. S4). While ofloxacin induced the SOS response in *E. coli*, zinc acetate concentrations of ≥0.4 mM significantly reduced the ofloxacin-induced SOS response in a concentration-dependent manner (Fig. S3). We also evaluated the synergistic effect of fluoroquinolones and zinc acetate against *E. coli*. The results showed that zinc acetate at concentrations of ≥1.0 mM only slightly decreased the MIC values of the fluoroquinolones ofloxacin and tosufloxacin (Fig. S4). Concentrations of zinc acetate higher than 1.2 mM inhibited the growth rate of the *E. coli* bacteria tested (Fig. S5).

We then tested the ability of three different concentration of zinc acetate (0.5 mM, 0.75 mM, and 1 mM) to potentiate the activity of the fluoroquinolones ofloxacin and tosufloxacin against biofilm persisters upon starvation. Zinc acetate was added at different times during the protocol to assess whether the inhibition of the SOS response at different stages would equally impact the efficacy of zinc acetate as an adjuvant treatment. To mimic potential clinical treatment options, zinc acetate was added after the biofilm was formed, during the posttreatment recovery phase, or at both times. We chose not to assess the adjuvant effect of zinc acetate during biofilm formation, since it would not be clinically applicable for use as an inhibitor prior to biofilm formation being detected. We observed that while zinc acetate alone did not impact the survival of biofilm cells (Fig. S6), its addition to ofloxacin and tosufloxacin treatments significantly but moderately reduced the levels of persisters in starved biofilms when it was added during only the treatment phase, during only the recovery phase, and during both the fluoroquinolone treatment phase and the posttreatment recovery phase (Fig. 4). Regarding potentiation of fluoroquinolone activity against stationary-phase bacteria, we determined that while zinc acetate alone did not impact the survival of

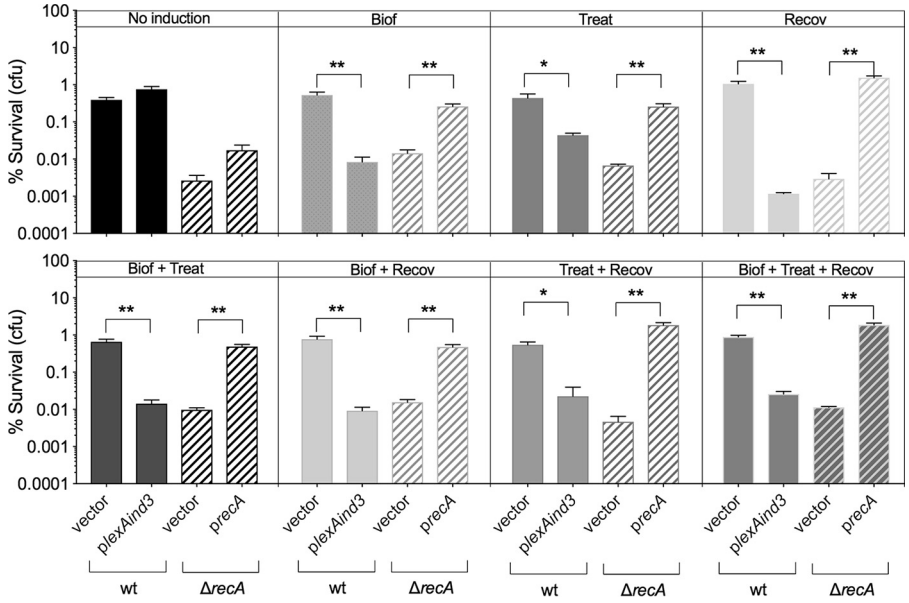

FIG 3 RecA and the SOS response are critical to ofloxacin persistence in starved biofilms, at least during the recovery phase. Biofilms of *E. coli* (TG1, plain bars, and TG1Δ*recA* [TG1Δ*recA*::FRT], hatched bars) were grown for 24 h in M63B1Gluc medium and treated for a period of 24 h with 80× MIC of ofloxacin in M63B1 medium without glucose (starved). Vector control corresponds to plasmid pUA66. The *recA* and *lexA3* (*lexAind3*) alleles were expressed from the P_T5-lac promoter. p*recA* corresponds to the pKV012 plasmid (pUA66 PT5*lac-recA*), and p*lexAind3* corresponds to the pKV013 plasmid (pUA66 PT5*lac-lexA3*). The number of ofloxacin persisters in starved biofilms was decreased in the absence of *recA* and following the induction of *lexA3*. The stage at which the induction of *lexA3* or *recA* by adding IPTG (1 mM) was performed is indicated above each set of histograms. Percentage (%) of survival represents the CFU count of viable cells after 24 h of treatment compared to that of untreated biofilms prior to addition of antibiotics. Each percentage (%) value is the mean plus or minus standard error of the mean (SEM) from at least three replicates. Asterisks indicate values significantly different from that of the control strain, which harbored the pUA66 plasmid. Biof, during biofilm treatment; Treat, during ofloxacin treatment; Recov, during recovery phase. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

stationary cells (Fig. S7), its addition significantly reduced the levels of persisters treated with ofloxacin and tosufloxacin when it was added during only the treatment phase, during only the recovery phase, and during both the fluoroquinolone-treatment phase and the posttreatment recovery phase (Fig. 5). Treatment with 80-fold the MIC of tosufloxacin in combination with 1.0 mM zinc acetate during both the treatment phase and recovery phase actually eradicated the *E. coli* persisters.

DISCUSSION

This study showed that zinc acetate inhibited the SOS response and enhanced fluoroquinolone activity, especially that of tosufloxacin, against *E. coli* biofilms and persisters. This combination (fluoroquinolone with zinc) is a potential strategy for treating biofilm-related bacterial infections.

Our study showed that, like the last-resort antimicrobial but highly toxic agent polymyxin B, fluoroquinolones, including tosufloxacin, were effective against *E. coli* persisters in biofilm, although most of the antibiotics tested in this study could not efficiently kill *E. coli*, especially in starved biofilms. Unlike in *E. coli*, Kobayashi et al. showed that tosufloxacin was relatively inefficient to kill *Pseudomonas aeruginosa* biofilm bacteria (18). The toxicity of tosufloxacin on articular cartilage is milder than that of the well-used fluoroquinolones norfloxacin and ciprofloxacin (19), and no other specific toxicity or enhanced toxicity was observed. Tosufloxacin is reported to display high clinical efficacy, especially for the treatment of chronic urinary tract infections (20, 21). To control chronic infections, including UTIs, that can be related to persisters in biofilms, tosufloxacin may be an effective choice.

We showed that starved biofilm tolerance was dependent on a functional SOS

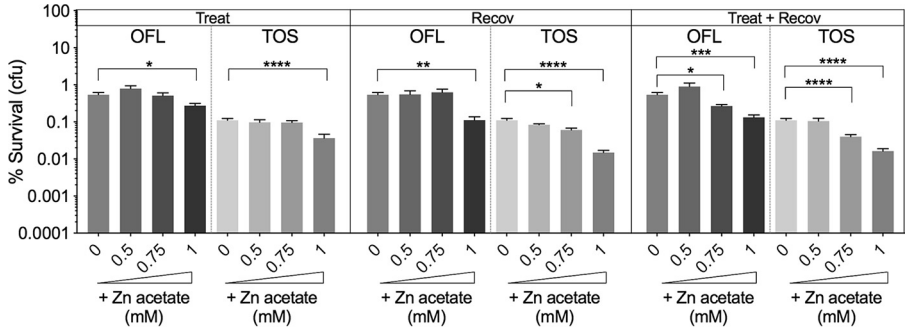

FIG 4 Zinc acetate decreases the survival rate of fluoroquinolone (ofloxacin and tosufloxacin)-treated starved biofilms. Biofilms of *E. coli* TG1 were grown for 24 h in M63B1Gluc medium and treated for a period of 24 h with 80× MIC of ofloxacin (5 mg/liter) or tosufloxacin (5 mg/liter) in M63B1 medium without glucose (starved). Zinc acetate was added during fluoroquinolone treatment only, during posttreatment recovery only, or during both fluoroquinolone treatment and posttreatment recovery. Each percentages of survival (%) value is the mean plus or minus standard error of the mean (SEM) from at least nine replicates. Asterisks indicate values significantly different from that of the fluoroquinolone-only treatment group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

response when treated by fluoroquinolones but not when treated by other classes of antibiotics, including beta-lactams, that are known to induce SOS response in *E. coli*. This points to other mechanisms of tolerance developed by *E. coli* to sustain these high antibiotic concentrations, such as possible growth arrest causing tolerance to beta-lactams (8). Unlike fluoroquinolones, biofilm tolerance to the quinolone nalidixic acid was relatively high and was not dependent on a functional SOS response. In addition to a potential lower capacity of nalidixic acid compared to that of fluoroquinolones to penetrate into biofilm matrix and biofilm cells, this difference could be explained mechanistically. While nalidixic acid treatment induces the SOS response, drug damages caused by this drug are repaired by the recombination repair system rather than by the SOS DNA repair system (22). In contrast, we and others have shown that tolerance to different fluoroquinolones is absolutely dependent on the SOS response. A study by the group of Kim Lewis has notably shown that SOS-dependent DNA repair genes, such as *dinG*, *ruvAB*, and *uvrD*, were directly necessary for *E. coli* tolerance to fluoroquinolones (23). Therefore, tolerance to nalidixic acid may be due to SOS-independent recombination repair, while tolerance to fluoroquinolones instead depends on SOS-dependent DNA repair.

Our previous results showed that starvation in biofilm bacteria induced an SOS-

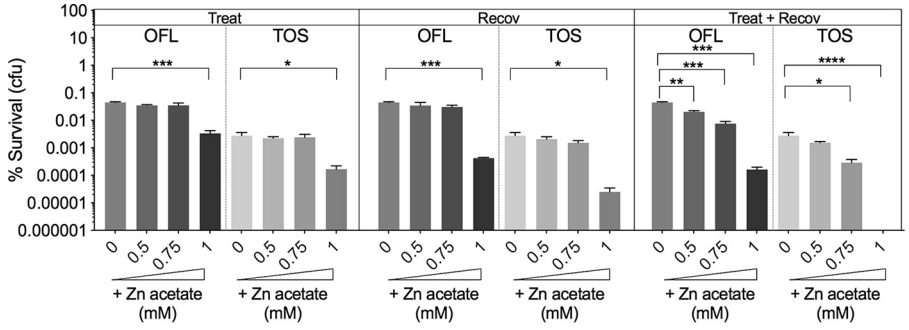

FIG 5 Zinc acetate decreases the survival rates of fluoroquinolone (ofloxacin and tosufloxacin)-treated *E. coli* in the stationary phase. *E. coli* TG1 cells were grown for 24 h in LB broth and treated for a period of 24 h with 80× MIC of ofloxacin (5 mg/liter) or tosufloxacin (5 mg/liter). Zinc acetate was added during fluoroquinolone treatment only, during posttreatment recovery only, or during both fluoroquinolone treatment and posttreatment recovery. Each percentage of survival (%) value is the mean plus or minus standard error of the mean (SEM) from at least three replicates. Asterisks indicate values significantly different from those of the fluoroquinolone-only treatment group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

dependent ofloxacin tolerance, but that it was independent of the toxin-antitoxin (TA) loci induced by the SOS response (8). Here, using different variants of LexA with gradually increased capacity to induce the SOS response, we demonstrated that the observed levels of tolerance were directly correlated with the levels of SOS induction. Additionally, we showed using *recA*- and *lexAind3*-expressing variants, in accordance with Völzing and Brynildsen (9), that SOS induction during the recovery phase was sufficient to increase the survival of biofilm bacteria treated by fluoroquinolone. In addition, we also showed that induction of the SOS response during biofilm formation or during treatment was also sufficient to promote increased survival of biofilm bacteria when exposed to fluoroquinolones.

Accordingly, we showed that the use of an SOS inhibitor, zinc acetate, in combination with tosufloxacin during the antibiotic treatment phase or during the posttreatment recovery phase efficiently killed persisters in starved biofilms and in the stationary phase. We did not evaluate the activity of zinc acetate combined with tosufloxacin during biofilm formation because of the absence of clinical relevance of this situation. While for biofilm treatment there was only a very slight increased gain of efficacy from adding zinc acetate both during treatment and recovery phase compared to only during recovery phase, this gain was much stronger when treating planktonic population and actually enabled eradication of *E. coli* persisters (80-fold MIC of tosufloxacin combined with 1.0 mM zinc acetate). A similar eradication of another *E. coli* strain was obtained in a different study using a higher concentration (333-fold MIC) of tosufloxacin (6). These results suggest that the combination of tosufloxacin and zinc acetate may reduce the required dose of tosufloxacin, the duration of antibiotic administration, and the toxicity of tosufloxacin. These results may also have some implications in term of treatment schedule.

The SOS response is involved in bacterial adaptive responses and horizontal gene transfer, potentially leading to the onset of antibiotic resistance in a broad range of bacterial species. Recent studies have shown that zinc is efficient at inhibiting both resistance mutation induced by quinolones in *E. coli* and *Klebsiella pneumoniae* (14) and SOS-induced development of chloramphenicol resistance in *Enterobacter cloacae* (12). Additionally, the same authors also showed that zinc was able to inhibit the transfer of an extended spectrum beta-lactamase (ESBL) gene from *Enterobacter cloacae* to *E. coli* (12). At much higher concentrations than the one used in our study, zinc has a direct antibacterial and antibiofilm activity (24). However, zinc toxicity in humans needs to be considered. Zinc acetate is commonly used in human for treating the common cold and zinc deficiency, and it is considered relatively nontoxic for humans. In these situations, it is delivered at the concentration of 150 mg/day with no detected side effect (25). The use of zinc acetate at 1 mM corresponds to 183 mg/liter and 65.3 mg/liter of zinc. This is comparable to zinc concentrations described in the literature to be toxic for humans and that can, for example, cause anemia or neutropenia, corresponding to 100 to 300 mg of zinc per day (25). Thus, it is possible to consider the use of a relatively low volume (5 to 100 ml) of 1 mM zinc acetate to locally help the treatment of biofilm-related infections. While further assays using different *in vitro* and *in vivo* models as well as clinical studies are necessary to evaluate efficacy and toxicity of zinc acetate in these situations, local adjuvant strategy of antibiotic treatment of infected wounds, recurrent UTI treatment by bladder instillation together with systemic antibiotic treatment, and the use of zinc acetate during or right after using conservative catheter-lock strategy could be considered in the future. Based on our findings, more specifically, in these clinical situations, a treatment of zinc acetate given concomitantly with the fluoroquinolone treatment or subsequently alone after the antibiotic treatment could reduce the formation of persisters during a potential regrowth of the biofilms. As we showed, using zinc acetate both during the fluoroquinolone treatment and after it, during recovery, may also improve treatment efficacy in targeting both biofilms and planktonic bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table S1. All experiments were performed at 37°C using M63B1 minimal medium containing 0.4% glucose (M63B1Gluc) or Miller's LB medium (Thermo Scientific, Rochester, NY) unless otherwise specified. Antibiotics were added, when required, at the following concentrations: kanamycin, 50 mg/liter; tetracycline, 15 mg/liter; chloramphenicol, 25 mg/liter; and streptomycin, 100 mg/liter. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

***E. coli* survival against several antibiotics in starved and nonstarved biofilms.** Test concentrations of the antibiotics used in survival assays were based on MIC values determined using microbroth dilution methods in M63B1Gluc, as previously described (8). The antibiotics tested were ofloxacin, ciprofloxacin, norfloxacin, nalidixic acid, amikacin, gentamicin, chloramphenicol, polymyxin B, rifampin, and tetracycline (all obtained from Sigma-Aldrich), ticarcillin (Ticarpen; GlaxoSmithKline, Marly le-Roi, France), and tosufloxacin (Wako Chemical, Tokyo, Japan). The MIC values for the strains of bacteria tested are shown in Table S2.

In the survival assays, *in vitro* biofilms were grown in triplicate for 24 h on UV-sterilized 96-well polyvinyl chloride (PVC) plates (Thermo Scientific) as previously described (8). Briefly, stationary-phase cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh M63B1Gluc, and 100 μl of this inoculum was used in each well. Biofilms were grown at 37°C in static conditions. After 24 h, each well was washed twice with 1× phosphate-buffered saline (PBS) to remove the unattached and planktonic bacteria. The remaining biofilms were treated for 24 h using 100 μl of M63B1Gluc (nonstarved) or M63B1 without glucose (starved), with or without antibiotics. The specific concentration used for each antibiotic was 80-fold its respective MIC against *E. coli* strain TG1, except for ticarcillin, for which a 100-fold MIC was used. After 24 h, each well was washed twice with 1× PBS to remove the antibiotic. Surviving bacteria were quantified after vigorous pipetting to detach all biofilm bacteria from the surface of the wells. After serial dilution, 10 μl per dilution of sample was spotted onto LB agar plates and incubated for 24 h at 37°C, and colonies were counted per sample. The total biofilm area cell density prior to antibiotic treatment was $4.9 \pm 2.7 \times 10^5$ CFU/mm^2. The CFU values were compared with those from untreated 24-h biofilms and expressed as the percentage of survival.

Time-kill curve of *E. coli* starved and nonstarved biofilms. *In vitro* biofilms were grown in triplicate for 24 h on UV-sterilized 96-well PVC plates (Thermo Scientific). Stationary-phase cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh M63B1Gluc, and 100 μl of this inoculum was used in each well. Biofilms were grown at 37°C in static conditions. After 24 h, each well was washed twice with 1× PBS to remove the unattached and planktonic bacteria. Twenty-four-hour biofilms were treated using 80-fold MIC of ofloxacin or tosufloxacin at 1, 2, 4, 8, 20, 24, and 48 h, after which each well was washed twice with 1× PBS to remove the antibiotics. Surviving bacteria were quantified after vigorous pipetting to detach all biofilm bacteria from the surface of the wells. The CFU values were compared with those from untreated 24-h biofilms and expressed as the percentage of survival.

***E. coli* survival against ofloxacin in planktonic stationary and starved biofilms with or without the SOS response.** To clarify the role of the SOS response in ofloxacin tolerance in the stationary phase of liquid culture and starved biofilms, we determined the survival rate of different strains of *E. coli* expressing different variants of *lexA*. Several *lexA* variants (MMR102-*lexA* wt, S119A, G80A, and E86P; see Table S1) previously described were kindly provided by Rahul M. Kohli (17). The wt and *lexA* variant-carrying strains received the F′tet plasmid through conjugation to favor biofilm formation (26, 27) (Table S1).

For testing the ofloxacin and ticarcillin tolerance in the stationary phase of liquid culture, the survival rates of these strains treated with ofloxacin/ticarcillin in the stationary phase of growth were determined as previously described (9), with some modifications. Briefly, these strain cultures were grown in 0.5 ml of Miller's LB medium overnight at 37°C with shaking (200 rpm). A 30-μl aliquot of this starter culture was used to inoculate 3 ml of Miller's LB medium and was subsequently grown at 37°C with shaking for another 16 h. To prepare cultures for ofloxacin/tosufloxacin treatment, 25 ml of Miller's LB medium were inoculated with overnight cultures and grown to an OD_{600} of 0.01. Cultures were then propagated for 20 h at 37°C with shaking. After 20 h, 500 μl of sample was collected, washed with PBS, and the CFU determined. The remaining samples were centrifuged, washed, and treated with 80-fold MIC of ofloxacin or 100-fold MIC of ticarcillin for 24 h in M63B1 medium. After the 24-h treatment, 500-μl samples were collected, washed by PBS, and the CFU determined. The CFU values were compared with those from untreated 24-h samples and the data expressed as the percentage of survival.

For testing the ofloxacin and ticarcillin tolerance in starved biofilm, the *lexA* variant-carrying strains received the F′tet plasmid through conjugation to favor biofilm formation (26, 27) (Table S1). The survival rates of the conjugated variants against ofloxacin and ticarcillin in starved biofilms were determined as described above.

The survival rates of *E. coli* TG1 carrying plasmids expressing *recA* or *lexAind3* (Table S1) in the presence of ofloxacin were determined as described above. Induction of *recA* or *lexAind3* was initiated using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) during several growth phases to determine when starved biofilm persisters in the presence of ofloxacin required the SOS response for survival. The phases considered included the biofilm formation phase, ofloxacin treatment phase, and posttreatment recovery phase. For the biofilm formation phase and ofloxacin treatment phase, 1 mM IPTG was added to the medium. For the posttreatment recovery phase, 10 μl per dilution of detached biofilm was spotted onto LB agar plates containing 1 mM IPTG and incubated for 24 h at 37°C, and colonies were counted per sample.

Evaluation of zinc acetate as an SOS response inhibitor by β-galactosidase assay. SOS induction was measured using an *E. coli* strain carrying the *sulA* SOS promoter fused to *lacZ* (p*sulA*::*lacZ*, JJC610) (28). The strain was cultured to an OD_{600} of 0.6 in LB medium. Ofloxacin (0.125 mg/liter) and several concentrations of zinc acetate (Sigma-Aldrich) ranging from 0 to 1 mM were added, and the cultures were incubated for an additional 1 h at 37°C. To determine the expression level of *sulA*, β-galactosidase activity was directly measured as previously described (29).

Susceptibility and survival of *E. coli* against ofloxacin/tosufloxacin with or without zinc acetate. The combination of the fluoroquinolone antibiotics ofloxacin and tosufloxacin with zinc acetate was evaluated using a checkerboard assay. The broth microdilution method used to determine the MIC values in 96-well microtiter plates was modified for the checkerboard assay. Briefly, 50 μl of M63B1Gluc was distributed into each well of the microtiter plates. The ofloxacin/tosufloxacin mixture was 2-fold serially diluted along the abscissa, while the zinc acetate was diluted along the ordinate. An inoculum equal to a 0.5 McFarland turbidity standard was prepared using *E. coli* TG1 in M63B1Gluc. Each microtiter well was inoculated with 50 μl of bacteria (5×10^4 CFU/well), and the plates were incubated at 37°C for 24 h.

The effect of zinc acetate on bacterial growth was determined by following the optical density of cultures (OD_{600}) using a Sunrise Rainbow Thermo microplate reader (Tecan, Kawasaki, Japan). *E. coli* TG1 cultures were grown in 0.5 ml of Miller's LB medium overnight at 37°C, and 1×10^5 CFU/ml bacteria were subsequently cultured in 0.1 ml of Miller's LB medium in 96-well plates at 37°C. The OD_{600} values were measured every 1 h for 12 h.

For the survival assays, the survival rates of *E. coli* TG1 against ofloxacin/tosufloxacin in starved biofilms were determined as described above. To clarify the effect of zinc acetate on the survival of persisters in starved biofilms treated with ofloxacin/tosufloxacin, several concentrations of zinc acetate were added to the cultures during the ofloxacin/tosufloxacin 24-h treatment phase and/or the post-treatment recovery phase. As a control we also assessed the effect of different concentrations of zinc acetate when added alone for 24 h after biofilm growth and during posttreatment recovery. For the posttreatment recovery phase, 10 μl per dilution of detached biofilm was spotted onto LB agar plates with or without several concentration of zinc acetate, and incubated for 24 h at 37°C, and colonies were counted per sample.

In addition, the survival rates of *E. coli* TG1 treated with ofloxacin/tosufloxacin in the stationary phase of growth were determined as previously described (9), with some modifications. Briefly, *E. coli* TG1 cultures were grown in 0.5 ml of Miller's LB medium overnight at 37°C with shaking (200 rpm). A 30-μl aliquot of this starter culture was used to inoculate 3 ml of Miller's LB medium and subsequently grown at 37°C with shaking for another 16 h. To prepare cultures for ofloxacin/tosufloxacin treatment, 25 ml of Miller's LB medium were inoculated with overnight cultures and grown to an OD_{600} of 0.01. Cultures were then propagated for 20 h at 37°C with shaking. After 20 h, 500-μl samples were collected, washed with PBS, and the CFU determined. The remaining samples were treated with 80-fold the MIC of ofloxacin/tosufloxacin for 24 h. After the 24-h treatment, 500-μl samples were collected, washed by PBS, and the CFU determined. The CFU values were compared with those from untreated 24-h samples and the data expressed as the percentage of survival. To determine the effect of zinc acetate on the survival of persisters treated with ofloxacin/tosufloxacin during the stationary phase, several concentrations of zinc acetate were added during the ofloxacin/tosufloxacin treatment phase and/or the posttreatment recovery phase. As a control we also assessed the effect of different concentrations of zinc acetate when added alone for 24 h on the stationary-phase cultures and during posttreatment recovery. For the posttreatment recovery phase, 10 μl per dilution of planktonic bacteria were spotted onto LB agar plates with or without several concentration of zinc acetate and incubated for 24 h at 37°C, and colonies were counted per sample.

Statistical analysis. Each experiment was performed at least three times. Statistical analysis was performed using a two-tailed unpaired *t* test. Differences were considered statistically significant for *P* values of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00069-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We have no conflicts of interest to declare.

REFERENCES

1. Lebeaux D, Ghigo GM, Beloin C. 2014. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev 78:510–543. https://doi.org/10.1128/MMBR.00013-14.
2. Stewart PS. 2015. Antimicrobial tolerance in biofilm. Microbiol Spectr 3. https://doi.org/10.1128/microbiolspec.MB-0010-2014.
3. Lewis K. 2001. Riddle of biofilm resistance. Antimicrob Agents Chemother 45:999–1007. https://doi.org/10.1128/AAC.45.4.999-1007.2001.
4. Kaper JB, Nataro JP, Mobley HL. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140. https://doi.org/10.1038/nrmicro818.
5. Foxman B. 2010. The epidemiology of urinary tract infection. Nat Rev Urol 7:653–660. https://doi.org/10.1038/nrurol.2010.190.
6. Niu H, Cui P, Shi W, Zhang S, Feng J, Wang Y, Sullivan D, Zhang W, Zhu B, Zhang Y. 2015. Identification of anti-persister activity against uropathogenic *Escherichia coli* from a clinical drug library. Antibiotics (Basel) 4:179–187. https://doi.org/10.3390/antibiotics4020179.
7. Niu H, Cui P, Yee R, Shi W, Zhang S, Feng J, Sullivan D, Zhang W, Zhu B, Zhang Y. 2015. A clinical drug library screen identifies tosufloxacin as being highly active against *Staphylococcus aureus* persisters. Antibiotics (Basel) 4:329–336. https://doi.org/10.3390/antibiotics4030329.
8. Bernier SP, Lebeaux D, DeFrancesco AS, Valomon A, Soubigou G, Coppée J-Y, Ghigo J-M, Beloin C. 2013. Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. PLoS Genet 9:e1003144. https://doi.org/10.1371/journal.pgen.1003144.
9. Völzing KG, Brynildsen MP. 2015. Stationary-phase persisters to ofloxacin sustain DNA damage and require repair systems only during recovery. mBio 6:e00731. https://doi.org/10.1128/mBio.00731-15.
10. Culyba MJ, Mo CY, Kohli RM. 2015. Targets for combating the evolution of acquired antibiotic resistance. Biochem 54:3573–3582. https://doi.org/10.1021/acs.biochem.5b00109.
11. Sexton JZ, Wigle TJ, He Q, Hughes MA, Smith GR, Singleton SF, Williams AL, Yeh L-A. 2010. Novel inhibitors of *E. coli* RecA ATPase activity. Curr Chem Genomics 4:34–42. https://doi.org/10.2174/1875397301004010034.
12. Crane JK, Cheema MB, Olyer MA, Sutton MD. 2018. Zinc blockade of SOS response inhibits horizontal transfer of antibiotic resistance genes in enteric bacteria. Front Cell Infect Microbiol 8:410. https://doi.org/10.3389/fcimb.2018.00410.
13. Nautiyal A, Patil KN, Muniyappa K. 2014. Suramin is a potent and selective inhibitor of *Mycobacterium tuberculosis* RecA protein and the SOS response: RecA as a potential target for antibacterial drug discovery. J Antimicrob Chemother 69:1834–1843. https://doi.org/10.1093/jac/dku080.
14. Bunnell BE, Escobar JF, Bair KL, Sutton MD, Crane JK. 2017. Zinc blocks SOS-induced antibiotic resistance via inhibition of RecA in *Escherichia coli*. PLoS One 12:e0178303. https://doi.org/10.1371/journal.pone.0178303.
15. Alam MK, Alhhazmi A, DeCoteau JF, Luo Y, Geyer CR. 2016. RecA inhibitors potentiate antibiotic activity and block evolution of antibiotic resistance. Cell Chem Biol 23:381–391. https://doi.org/10.1016/j.chembiol.2016.02.010.
16. Wigle TJ, Sexton JZ, Gromova AV, Hadimani MB, Hughes MA, Smith GR, Yeh LA, Singleton SF. 2009. Inhibitors of RecA activity discovered by high-throughput screening: cell-permeable small molecules attenuate the SOS response in *Escherichia coli*. J Biomol Screen 14:1092–1101. https://doi.org/10.1177/1087057109342126.
17. Mo CY, Manning SA, Roggiani M, Culyba MJ, Samuels AN, Sniegowski PD, Goulian M, Kohli RM. 2016. Systematically altering bacterial SOS activity under stress reveals therapeutic strategies for potentiating antibiotics. mSphere 1:e00163-16. https://doi.org/10.1128/mSphere.00163-16.
18. Kobayashi H, Ohgaki N, Takeda H. 1993. Therapeutic possibilities for diffuse panbronchiolitis. Int J Antimicrob Agents 3:S81–S86. https://doi.org/10.1016/0924-8579(93)90039-8.
19. Furubo S, Fukuda H, Kozaki T, Akune A, Sanzen T. 2010. Toxicity studies of tosufloxacin tosilate hydrate in juvenile animals and class effects studies. Jpn J Chemother 58:12–23.
20. Sakurai M, Sugimura Y, Arima K, Kawamura J, Saitou K, Tajima K, Nakano S, Satani H, Nagano M, Hoshina A. 1994. Study on clinical effects of tosufloxacin (TFLX) and the long-term low dose therapy for the prophylaxis of recurrent urinary tract infection. Hinyokika Kiyo 40:279–284.
21. Yoshida K, Kobayashi N, Saitoh H, Negishi T, Yamada T, Watanabe T, Kawakami S, Tari K, Satake I, Ozawa K. 1992. Clinical efficacy of tosufloxacin on the patients with urinary tract infections. Hinyokika Kiyo 38:129–134.
22. Lewin CS, Howard BM, Ratcliffe NT, Smith JT. 1989. 4-Quinolones and the SOS response. J Med Microbiol 29:139–144. https://doi.org/10.1099/00222615-29-2-139.
23. Theodore A, Lewis K, Vulic M. 2013. Tolerance of *Escherichia coli* to fluoroquinolone antibiotics depends on specific components of the SOS response pathway. Genetics 195:1265–1276. https://doi.org/10.1534/genetics.113.152306.
24. Gugala N, Lemire JA, Turner RJ. 2017. The efficacy of different anti-microbial metals at preventing the formation of, and eradicating bacterial biofilms of pathogenic indicator strains. J Antibiot 70:775–780. https://doi.org/10.1038/ja.2017.10.
25. Fosmire GJ. 1990. Zinc toxicity. Am J Clin Nutr 51:225–227. https://doi.org/10.1093/ajcn/51.2.225.
26. Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, Haagensen JA, Molin S, Prensier G, Arbeille B, Ghigo JM. 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol Microbiol 51:659–674. https://doi.org/10.1046/j.1365-2958.2003.03865.x.
27. Ghigo JM. 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature 412:442–445. https://doi.org/10.1038/35086581.
28. Flores MJ, Sanchez N, Michel B. 2005. A fork-clearing role for UvrD. Mol Microbiol 57:1664–1675. https://doi.org/10.1111/j.1365-2958.2005.04753.x.
29. Miller A. 1992. Short course in bacterial genetics – a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.